

USING THE BACTERIA-2-HYBRID SYSTEM TO DETERMINE
THE ROLE OF S2 IN THE EQUINE INFECTIOUS ANEMIA
VIRUS (EIAV) LIFE CYCLE

A Senior Honors Thesis

by

JASON LEE WEIDNER

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
In partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2003

Group: Life Sciences II

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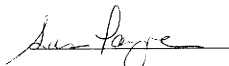
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ABSTRACT

Using the Bacteria-2-Hybrid System to determine the role of S2 in the Equine Infectious Anemia Virus (EIAV) Life Cycle. (April 2003)

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After in vivo and in vitro experiments comparing wild type and S2 knock-out strains of EIAV were conducted, the role of one small accessory protein still eluded discovery, S2. S2, is a small protein that has a nuclear localization sequence, SH3 binding domain ligand, and is only 19 kD in size. It was used in a bacterial two-hybrid to discover interacting host cell partner proteins from a mouse lung cDNA library via transcriptional activation.

The experiment used a genetically modified strain of *E. coli* to test interactions between fusion products made by fusing S2 with lambda repressor, and random cellular proteins to RNA polymerase (RNAP). Since a reporter strain cassette is contained within the genetically altered bacteria, the lambda repressor can bind to the altered lambda operator, freeing the S2 particle for interaction. If a single bacteria has the plasmid that codes for S2 and the interacting partner, then the interacting partner can bind to S2, guiding RNAP to transcribe the reporter gene, carbenicillin. The experiment

used plates with differing carbenicillin concentrations to test for stronger protein-protein interactions, and has resulted in about thirty different putative positive partners.

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INTRODUCTION

The Biology of Equine Infectious Anemia Virus

Equine Infectious Anemia Virus (EIAV) is a retrovirus of the genus lentivirus along with the human immunodeficiency viruses (HIV-1, HIV-2), simian immunodeficiency virus and feline immunodeficiency virus. The clinical signs of equine infectious anemia (EIA) usually show themselves anywhere from 6 to 30 days post infection (Kono et al., 1969, Kemeny et al., 1971) and include febrile episodes with viremia (Kono et al., 1969). The high temperature is partly due to the increase in cytokines such as TNF and IL-1 levels released upon destruction of macrophages (Cook et al., 1996), the cells in which EIAV replicates. EIAV replication is thought to start in cells of the macrophage/monocyte lineage at the site of inoculation. Eventually, disease spreads throughout the body infecting only the monocyte/macrophage cells, and the Kupffer cells of the liver. The clinical signs of EIA can vary from very mild or inapparent infection to death. During the acute stage of infection, the disease episodes usually decline in severity until the animal becomes an inapparent carrier (Kono et al., 1974a, Kono et al., 1974b). In addition to fever, and multiple disease episodes, symptoms can include small lesions on mucous membranes, depression, anorexia and weight loss.

The genome of EIAV is 8200 nucleotides long and includes *gag*, *pol*, S1, S2, *env*, and S3 open reading frames. Long Terminal Repeat sequences (LTRs) flank each end of the genome (see figure 1). The *gag* and *env* genes encode proteins that make up the virus

This thesis follows the style and format of *Virology*.

particle, while S1 and S3 encode accessory proteins. The S1 gene encodes a protein that has been assigned the name *tat*. *Tat* comes from the transactivator protein in HIV, where HIV's *tat* protein reacts with the LTRs to increase the number of viral transcripts (7). The S3 region in EIAV encodes a protein that has been assigned the name *rev*. *Rev* comes from the HIV *rev* protein that inhibits splicing, a critical step in virus production (Noiman et al., 1990, Carroll et al., 1991). The role of S2 in the virus lifecycle is still unknown and is the focus of my studies.



Fig. 1. EIAV genome organization. The *gag*, *pol*, and *env* are typical genes found in retroviruses. *Tat* and *rev* are common to all lentiviruses while, S2 is unique to EIAV.

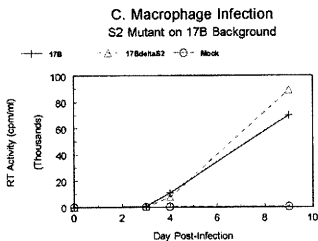


Fig. 2. This graph shows the growth properties of wild-type and S2 mutant EIAV in cell culture. Virus growth is monitored by reverse transcriptase (RT) activity. The red line represents the wild-type strain, while the green line represents the S2 mutant.

The EIAV S2 gene

Initial experiments to examine S2 function included a mutation of the gene on the background of an infectious molecular clone of EIAV. An *in vitro* comparison of the replication kinetics of the parental clone with the S2-knockout showed that the replication kinetics of the two strains were similar in equine macrophages (see figure 2). However the outcome of an *in vivo* experiment comparing the ability of these viruses to cause disease was significant. The wild type strain caused lethal disease while the mutant strain caused no disease (data not shown).

Even though S2 has not been fully characterized at the molecular level, possible functions have been suggested, based largely on similarities to proteins from HIV and SIV. One such hypothesis is that S2 might be a homologue of the *vpu* gene of other lentiviruses (Li et al., 2000). Even though S2 does not have a hydrophobic membrane spanning domain, or a CKII site (the site where *vpu* is phosphorylated by Casein Kinase II), it does have high threonine and serine residue content, which suggests that S2 may be phosphorylated. The location of the S2 gene in the EIAV genome is similar to the location of the *vpu* gene in the viral genome. The *vpu* gene has been shown to be involved in virion assembly and release (Montelaro et al., 1993, Strebel et al., 1989, Terwilliger et al., 1989). Another hypothesis as to the function of S2 is that it might play a similar role as *vpr* (Li et al., 2000). *Vpr* is a gene found in both SIV and HIV-1, where it stops host cell cycle and drives host cell differentiation, while up regulating viral gene expression (Cohen et al., 1990, Heizinger et al., 1994, Levy et al., 1993). The connection

was made between S2 and *vpr* mainly because they both have nuclear localization sequences on their C-terminus (Li et al., 2000). This second hypothesis causes controversy because S2 does not cause monocytes to differentiate into macrophages, nor does it affect virus infectivity *in vitro* (Li et al., 2000).

Identification of protein-protein interactions by two-hybrid screening

The lack of an observable phenotype for the S2 mutation in cell culture leads to an important question: How do we elucidate the function of a protein that seems to have an observable phenotype only *in vivo*? One way to do this is with a bacterial two-hybrid screen.

The **two-hybrid technique** is based on transcriptional activation, and is a variation of a normal cellular protein-protein interaction between RNA polymerase (RNAP), and a DNA binding protein (DNABP). Instead of using a typical RNA polymerase, and DNA binding proteins, these two proteins have been genetically altered to test interactions between a protein of interest and a test protein.

Bacterial 2-hybrid Reporter strain and Reporter cassette

The bacterial **two-hybrid system** is based on the protein interaction between RNAP, and a DNABP to transcribe a reporter gene. Instead of using a typical RNA polymerase, and DNA binding proteins, these two proteins have been genetically altered to test interactions between a protein of interest and a test protein. The test for protein – protein interactions requires that the cell not transcribe the reporter gene with its normal DNABP and RNAP. To ensure this is the case, the cells used in the experiment are a special reporter strain derived from XL1-Blue MR bacteria (Stratagene), and contains the

lacI^q gene to suppress the expression of the pBT and pTRG plasmids. Even though the pBT and pTRG are being suppressed by *lacI^q*, it should be noted that there is always a basal level of expression. Besides having *lacI^q*, the reporter strain also contains a reporter cassette that codes for ampicillin resistance and B-galactosidase. These reporter genes are located on the F' episome, and are under the control of a modified *lac* promoter. The modified *lac* promoter has a single lambda operator located at -62 so that it replaces the normal cAMP receptor protein (CRP)-binding site. The single lambda operator is the site of binding for the lambda repressor, which is the DNABP used in this screen.

To test the interaction between S2, and a test protein, S2 needs to be fused to the DNABP. To do so, S2 has a polyglycine linker added to it before it is cloned into the pBT plasmid, the plasmid that codes for DNABP. To decrease the number of false positives, the S2-linker needs to be cloned into pBT so that it is added to the N-terminus of the DNABP. By having the bait protein (S2-linker) fused to the N-terminus of the DNABP, the dimerization sequence of the DNABP that resides on the N-terminus of the DNABP, which usually interacts with the C-terminus of the RNAP, was not available for interaction. This means that if there is an interaction, the interaction is less likely to be from an unwanted interaction between the DNABP and RNAP.

The other component of the two-hybrid is the test protein. To see which equine macrophage proteins interact with S2, a cDNA library is made. The resulting cDNA fragments are into pTRG directionally so that the partner proteins are fused to the N-terminus of RNAP. This hinders dimerization sequence on the N-terminus of the RNAP resulting in fewer false positives.

primer I

GGAGCGGAGGTTCCGGAGTATTGGTA
 ATGGGAGTATTTGGTAAAGGGTAACATGGTCAGCATCGCATTCTATGGGGGAATCCCAGGG
 GGAATCTCAACCCCTATTACCAACAGTCAGAAAAATCTAAGTGTGAGGAGAAACAATGTTTC
S2
 AACCTTATTGTTATAATAATGACAGTAAGAACAGCATGGCAGAAATCGAAGGAAGCAAGAGACC
 GGCAGAAATCGAAGGAAGCAAGAGACC
 AAGAAATG **reverse primer**
 AAGAAATGAGGATCCATGCACGCC

↓ **PCR, gel purification, TOPO-cloning**

primer II

CGGAGGTGGAAGCGGTGGAG
 GCGGAAGTGGAGGCGGAGGT
 GGAGCGGAGGTTCCGGAGTATTGGTAAAGGGTAACATGGTCAGCATCGCATTCTATGGG
 GGAATCCCAAGGGGAATCTCAACCCCTATTACCAACAGTCAGAAAAATCTAAGTGTGAGGA
S2
 GAACACAATGTTTCAACCTTATTGTTATAATAATGACAGTAAGAACAGCATGGCAGAAATCGAAG
 GGCAAGATCGAAG
 GAAGCAAGAGACCAAGAAATGAGGATCCATGCACGCC
 GAAGCAAGAGACCAAGAAATGAGGATCCATGCACGCC **reverse primer**

↓ **PCR, gel purification, TOPO-cloning**

primer III

CGCATCCGAATTCAGGCGGAGGTGGAA
 CGGAGTGGAAAGCGGTGGAGGCGGAAGTGGAGGCGGAGGTTCCGGAGTATTGGTAAAG
 GGGTAACATGGTCAGCATCGCATTCTATGGGGGAATCCAGGGGGAATCTCAACCCCTATT
S2
 ACCCAACAGTCAGAAAAATCTAAGTGTGAGGAGAACACAATGTTTCAACCTTATTGTTATAATA
 ATGACAGTAAGAACAGCATGGCAGAAATCGAAGGAAGCAAGAGACCAAGAAATGAGGATCCA
 GGCAGAAATCGAAGGAAGCAAGAGACCAAGAAATGAGGATCCA
 TGCACGCC **reverse primer**
 TGCACGCC

↓ **PCR, gel purification, TOPO-cloning**

CGCATCCGAATTCAGGCGGAGGTGGAAAGCGGTGGAGGCGGAGGTTCCGGAGGCGGAGGTTCCG
 GAGTATTTGGTAAAGGGTAACATGGTCAGCATCGCATTCTATGGGGGAATCCCAAGGGGAAG
S2
 TCTCAACCCCTATTACCAACAGTCAGAAAAATCTAAGTGTGAGGAGAACACAATGTTTCAACC
 TTATTGTTATAATAATGACAGTAAGAACAGCATGGCAGAAATCGAAGGAAGCAAGAGACCAAGAA
 ATGAGGATCCATGCACGCC

Fig. 3. PCR strategy. Adding the linker on step-wise requires performing PCR, purifying the fragment, then cloning the fragment into the TOPO vector for use in the next round of PCR.

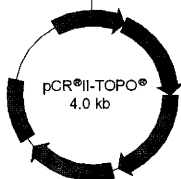


Fig. 4. The pCRII-TOPO vector has topoisomerase on each end insertion region where the PCR product ligates into the multiple cloning site. The topoisomerase grabs the poly-A tails left on the PCR product from the taq polymerase, and aids in the cloning of the PCR product into the vector.

Materials and Methods

Construction of the S2-DNABP fusion product

A flexible polyglycine linker was added in between the DNABP and S2. PCR was used to add the poly-gly linker to the N-terminus of S2 before cloning the S2-linker gene into the TOPO vector (see fig 3 <for the PCR strategy>, and figure 4 <for a restriction map of the PCRIL-TOPO plasmid>). The PCR reactions used one of three forward primers (depending on the step of poly-gly linker synthesis) and the same reverse primer. The first reaction consisted of 10 μ mol forward primer 1, 10 μ mol reverse primer, 0.25 μ L Taq polymerase, 1.0 μ L dNTPs, 4.0 μ L 10X reaction buffer containing Mg^{2+} , 34 μ L dH_2O , and <1 ng pET30-S2 as the template. The second and third reactions were kept the same except the forward primer. The PCR product formed in each round was used for the template of the next PCR reaction. After each PCR, the reaction mix was run on a 1% agarose gel, the PCR product was gel purified (QIAquick gel extraction kit by Qiagen), and the purified product was used as the template for the next PCR reaction.

To clone S2 into the TOPO vector the protocol included with the kit (TOPO TA Cloning kit dual promoter #K465040 by Invitrogen) was used. This required taking gel purified PCR product, incubating it with the chemically competent (came with the kit) on ice for 5 minutes, heat shocking the cells for 30 seconds at 42°C, adding 900 μ L SOC, shake at 37°C for 1 hour, then plating ~100 μ L on an LB-kan+X-gal plate. Plates were incubated the over-night at 37°C.

The next day, 3 mL of fresh LB-kan broth was inoculated with one white colony from the plate, incubated at 37°C, and shaken at 250 rpm over night. Plasmid DNA was isolated using a miniprep kit (Qiaprep Spin Miniprep Kit, Qiagen), and the purified plasmid was used as the template for the next PCR reaction.

With the insert in the TOPO plasmid, the TOPO-S2 clones were grown up in some in over-night cultures, and had their plasmids isolated via miniprep (Qiaprep spin miniprep kit, Qiagen). The plasmids were then digested with restriction enzymes BamHI and EcoRI (these sites flanked the S2-linker fragment). After the digestion, the digestion mixture was run on a 1% agarose gel, and the fragment containing the S2-linker sequence was gel purified (Qiaquick gel extraction kit by Qiagen) so that it could be ligated into some pBT plasmid that had also been cut with BamHI and EcoRI (see fig 5).

Construction of the cDNA library

A previously constructed cDNA library was used in the 2-hybrid screen. It was constructed as follows: The mRNA was isolated and purified from mouse lung cells. Then, cDNA was synthesized using reverse transcriptase, so that the cDNA could be cloned into pTRG, the plasmid that codes for RNAP. The pTRG plasmid confers tetracycline resistance (see figure 6).

Expression of pBT-S2 and Western Analysis

After pBT-S2 was constructed, the clone needed to be expressed to ensure that the fusion product was available for interaction in the cell. First, 3mL of fresh LB liquid media were inoculated with one colony of pBT-S2 and another 3mL of fresh LB liquid media with one colony of pBT. These were grown over-night at 30° C, shaking at 250 rpm. In the morning, separate 2mL aliquots of fresh LB and 15 uM IPTG were inoculated with a loop of each of the over night cultures, left incubating for 6 hours at 30° C, while shaking at 250 rpm. Then the cells were prepared for analysis by SDS-PAGE by spinning the cells at 10,000 rpm for 2 minutes, pouring off the supernatant, and adding SDS-PAGE sample buffer. Then the induced culture, uninduced culture and purified recombinant S2 protein samples were loaded onto two different 15% SDS-PAGE gels, and run at 200mV for approximately an hour. Once ran, each gel was used to transfer the bands of proteins onto nitrocellulose. The transfer was conducted at 200 mV for 90 minutes. Once the proteins were transferred to nitrocellulose, membranes were blocked for 30 minutes with 5% non-fat dry milk + tris/saline (BLOTTO). Then the blots were washed with an anti-S2 antibody 1:1000 and EIAV infected horse sera

1:500 and BLOTTO mixture, and incubated for an hour at room temperature. The blots were then washed 5 minutes with tris/saline, 10 minutes tris/saline + 0.05% NP40, 10 minutes with tris/saline + 0.05% NP40, and 5 minutes tris/saline all at room temperature. Then the secondary antibody was bound to the blots by adding anti-horse conjugated horse radish peroxidase (HRP) 1:2000 in BLOTTO to one blot, and anti-mouse HRP 1:3000 in BLOTTO to the other. Chemoflourmetric substrate was then added to the blot to illuminate the bands with the HRP bound, and exposed film for one minute (see figure 7). Since the Western blot using the mouse-anti-S2 showed similar induction of S2 for both pBT and pBT-S2 induced clones, I decided to do another western, and ensure that the primary antibody is working. I loaded induced pBT, induced pBT-S2, uninduced pBT-S2, and purified prokaryotically expressed S2 onto a 15% SDS-PAGE gel, and ran at 200mV for approximately an hour. For the new western, pre-immune sera was used as the source of primary antibody (1:1000) and anti-mouse HRP (1:3000) and the secondary anti-body. Other than the primary anti-body, all of the western procedure were kept the same (see figure 8). As figure XX shows, there was no binding of the primary anti-body. This means that the binding from the Western using mouse-anti-S2 was non-specific. I am currently working on the expression of pBT-S2, checking that the positive controls in the western show positive results so that there is no doubt about the availability of S2 for interaction due to pBT-S2 not being expressed.

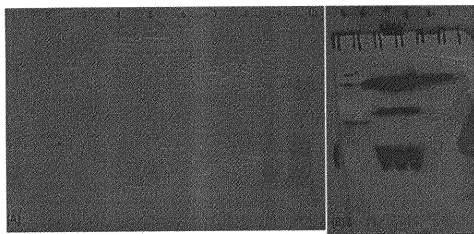


Fig. 7. Western blot analysis of induction of pBT-S2. (a) 1° anti-body EIIV infected horse sera (1:500), 2° anti-horse HRP (1:2000) 1. Multimark prestained standard (invitrogen) 2. purified prokaryotically expressed S2 from pET30-S2 3. purified prokaryotically expressed S2 from pET30-S2 4. induced pBT clone #3 5. induced pBT clone #4 6. empty 7. uninduced pBTS2 clone 6 8. induced pBTS2 clone 6 9. uninduced pBTS2 clone 6 10. multimark prestained marker (b) 1° anti-body mouse anti-S2 (1:1000), 2° anti-mouse HRP (1:3000) Assuming there is not non-specific binding from the mouse sera, S2 protein appears to be in several induced and uninduced samples. Gel : multimark prestained protein marker (invitrogen) 1. purified S2 from prokaryotically expressed pET30-S2 2. purified S2 from prokaryotically expressed pET30-S2 3. induced pBT 4. induced pBT 5. induced pBTS2 clone 6 6. uninduced pBTS2 clone 6

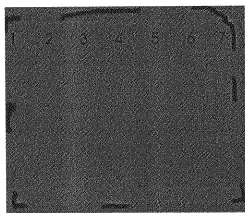


Fig. 8. Western blot with 1° antibody being pre-immune mouse sera, 2° anti-mouse HRP. 1. purified prokaryotically expressed S2 2. purified prokaryotically expressed S2 3. pBT clone #3 induced 4. pBT clone #4 induced 5. pBT-S2 clone #6 induced 6. pBT-S2 clone #6 uninduced 7. multimark prestained protein marker. No bands appearing means that there is not non-specific binding from the pre-immune mouse sera.

Table 1.
Essential controls for the bacterial 2-hybrid screen.

DNA used in transformation	Explanation of control	Antibiotics used
pBT-S2 only	Ensures pBT-S2 does not interact with E. coli RNAP	Kan, Cam, Tet, Carb
pTRG-library	Ensures pTRG-library does not interact with E. coli DNABP	Kan, Cam, Tet, Carb
pBT-S2+pTRG	Ensures pBT-S2 does not interact with phage RNAP	Kan, Cam, Tet, Carb
pControl	Ensures the transformation protocol worked	Cam

Cotransformation and screening

Cotransformation was performed using the BacterioMatch™ Reporter Strain Competent Cells (Stratagene). To grow the transformants, Luria Broth plates containing carbenicillin (250, 300, 350 and 400 ug/mL), chloramphenicol (10 ug/mL), tetracycline (15 ug/mL), and kanamycin (50 ug/mL) were used (LB-CTCK plates). The kanamycin and carbenicillin resistance is coded for by the reporter cassette, while the chloramphenicol and tetracycline resistances are coded for by the pBT and pTRG plasmids, respectively.

Several controls were conducted to ensure that positive colonies were not false positives (see table 1). The transformation of the reporter strain with pBT-S2 and pTRG-library separately ensures that neither the pBT-S2 fusion product or any of the pTRG-library test fusion products produce any colonies. If pBT-S2 produced colonies it would mean that the fusion product interacts with the regular host RNAP strongly enough to express carbenicillin resistance. If, on the other hand, pTRG-library had produced colonies, it would mean that at least some of the fusion products, made by the cDNA library, allowed the RNAP portion of the fusion product to read, and transcribe the reporter cassette's carbenicillin resistance gene. The appearance of colonies from either of these transformations would be detrimental to the project because it would mean that there were inherent complexities within either S2 or the cDNA library that would keep that component from being used in the bacterial-2-hybrid screen.

The transformation of the reporter strain with pBT-S2 and pTRG (without library) tests for an interaction between S2 and the RNAP coded for by pTRG. If

colonies result from this transformation it would mean that S2 interacts with the RNAP strongly enough to express the carbenicillin resistance gene on the reporter cassette. Again, positive colonies with this transformation would be devastating to the project. For the test interaction between the library fusion products and S2-DNABP to be legitimate, it is assumed that S2 does not interact with the test partner's adjacent RNAP.

One major caveat to the bacterial-2-hybrid system is the number of false positives. False positives come from unwanted interactions from S2-RNAP interactions, partner-DNABP interaction, or even DNABP-RNAP interactions. Luckily, not all of these interactions are strong interactions. By plating the transformations on LB-CTCK plates of differing carbenicillin concentrations, we can test for interactions of different strengths. This means that if the number of colonies from the pBT-S2 and pTRG-library test are many when compared to the number of colonies from negative controls, the experiment is not ruined. At that point, the results should be kept in perspective. If there are approximately one-tenth as many colonies on the negative controls as on the plate of the pBT-S2 and pTRG-library interaction, then one-tenth of the pBT-S2 and pTRG-library colonies are probably false positives.

To perform the cotransformation, reporter strain competent cells were thawed on ice, aliquoted out 62.5 μ L of cells into pre-chilled tubes, and had 1.2 μ L of β -mercaptoethanol added to each tube. Then the cells were incubated on ice for 10 minutes, and had 10 ng of each plasmid necessary for the experiment added, while incubating the cells on ice for an additional 30 minutes. After the 30 minutes, the cells

were heat shocked for 45 seconds at 42° C and incubated on ice for an additional 2 minutes. Then 900 uL of SOC were added to the cell mix so that the cells could grow up for 3 hours at 30°C. While the cells were growing, they were shaken at 250 rpm, just to be have 120 uL of cells plated on an LB-CTCK plate.

When the experiment was performed, the putative positive colonies needed to go through some processing. First, a master plates of putative positives were made (LB-CTCK with the carbenicillin concentration the same as the plate the positive colony came from), and then the pTRG-partner plasmid from each putative positive was isolated. That allowed a cotransformation using the purified pTRG-partner plasmid and pBT-S2 to be done. This cotransformation ensures that the pTRG-partner plasmid is a positive.

Of the putative positives that grow up after the second cotransformation, those pTRG-partner plasmids are cotransformed with pBT (without S2). This test will help to eliminate a family of false positives from the group of putative positives left from the second cotransformation.

To get the pTRG-partner plasmid from putative positives, a colony was taken from each putative positive and used to inoculate 3 mL LB-CTK liquid media to grow overnight. The purpose of leaving the chloramphenicol out of the media was to encourage the cells to lose the pBT-S2 plasmid. Once grown up, an inoculating loop full of overnight culture was used to inoculate another 3 mL of fresh LB-CTK media to grow up again overnight. This second overnight culture without chloramphenicol ensures that the pBT-S2 plasmid was lost. Once grown, the cells were pelleted by

spinning them at 10,000 rpm for 2 minutes. Then the supernatant was poured off, and the pTRG-partner plasmids were isolated via miniprep (Qiaquick spin miniprep kit, Qiagen).

pBT cut with
XbaI/XhoI
943, 2257

S2 -> ~200bp
linker -> ~50 bp

pBT with insert
cut with
XbaI/XhoI
should have a
~300 bp frag.

500 bp ----->

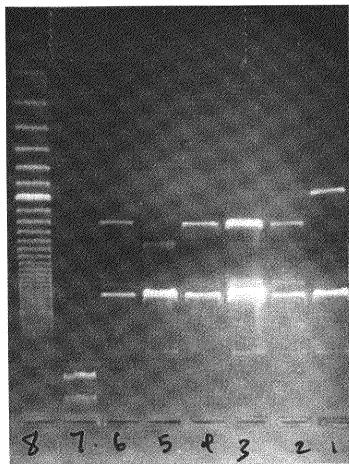


Fig. 9. 1% agarose gel of XbaI/XhoI digestions of pBT-S2 clones. 1) digest of pBT (no insert) 2) digest of pBT-S2 #2 3) digest of pBT-S2 #3 4) digest of pBT-S2 #4 5) digest of pBT-S2 #5 6) digest of pBT-S2 #6 7) control DNA (10 ng/uL) 8) 100 bp ladder All clones have insert except in lane 1 and 5. Lane 1 has no insert, while lane 5 has two inserts.

Results

cDNA Library Construction

Because equine macrophages, the regular host cell of EIAV, are difficult to grow in cell culture, the cDNA library was made from mouse lung cells. Lung cells were chosen because of the usual abundance of macrophage in that tissue.

Construction and Expression of pBT-S2

To ensure that S2 would be available for interaction, a polyglycine linker was engineered in frame and upstream of the S2 gene, before cloning S2 into pBT. The poly-gly linker was added in a stepwise fashion to complete the addition of the entire linker.

Each round of PCR required me to clone purified product into the PCR II-TOPO plasmid, plate transformants, grow up overnights and start over again using the new TOPO-S2 as a template for the next round of PCR. After the three rounds of PCR had been preformed, the TOPO-S2 was digested with EcoRI and BamHI, run on a 1% agarose gel, and the S2-linker DNA fragment was gel purified (QIAquick gel extraction kit by Qiagen), and ligated into EcoRI and BamHI digested pBT plasmid I using the Rapid DNA Ligation Kit (Boehringer Mannheim). When cloned into pBT, I confirmed S2 was in frame by DNA sequencing (see figure 9, 10).

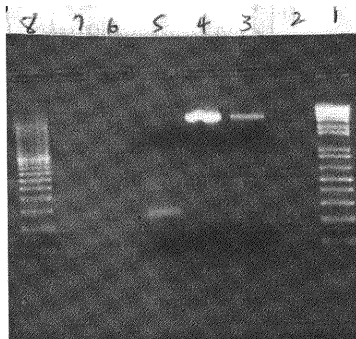


Fig. 10. 1% agarose gel of 1) 1 kb ladder 2) empty 3) uncut pBT 4) ~3.9 kb fragment of TOPO-S2 cut with EcoRI and BamHI 5) ~225 b fragment from TOPO-S2 cut with EcoRI and BamHI (presumably S2-linker) 6) empty 7) empty 8) 100bp ladder

Table 2

Number of colonies resulting from cotransformation.

# colonies	Interaction tested	250 ug/mL* Carbenicillin	300 ug/mL* Carbenicillin	350 ug/mL* Carbenicillin	400 ug/mL* Carbenicillin
Controls	pBT+pTRG	0	NA	NA	NA
	pTRG-library	0	NA	NA	NA
	pBT-S2+pTRG	0	NA	NA	NA
1.5 hour* incubation	pBT-S2+pTRG-library (1:1 dilution)	18	50	4	0
	pBT-S2+pTRG-library (1:10 dilution)	1	0	0	0
	pBT-S2+pTRG-library (1:100 dilution)	0	0	0	0
3 hours* incubation	pBT-S2+pTRG-library (1:1 dilution)	Not preformed	59	0	0
	pBT-S2+pTRG-library (1:10 dilution)	Not preformed	0	0	0
	pBT-S2+pTRG-library (1:100 dilution)	Not preformed	0	0	0

*Cotransformation with 10ng of appropriate DNA. DNA was added to 100 μ l aliquots of competent cells, incubated on ice for 30 minutes, and then heat-shocked at 42° for 45 seconds. After the heat shock, 900 μ l of SOC was added to each transformation reaction, and the cells were incubated at 30°C, shaking at 250 rpm for either 1.5 or 3 hours. 100 μ l of cells from each transformation except the pBT-S2 +pTRG-library were plated. The pBT-S2 +pTRG-library had 100 μ l volumes of cells from 1:1, 1:10, 1:100, and 1:1000 dilution plated on the plates of different carbenicillin concentration. The stronger carbenicillin tests for stronger interactions between S2 and the random cellular protein partners. NA means not applicable.

Cotransformation into Reporter Strain

With the necessary components constructed, I cotransformed a reporter strain that coded for carbenicillin resistance, and plated the cells on plates with differing carbenicillin concentrations (see table 2). By plating on plates of differing carbenicillin concentrations, I tested for protein-protein interactions of different strengths. The results are promising due to two main points: the negative results of the pBT-S2+pTRG control, and the increase in the number of colonies in the pBT-S2+pTRG-library (1:1 dilution, 1.5 hours of incubation) when plating on LB-CTCK plates where the carbenicillin was increased to 300 ug/mL. I took a colony from each putative positive and used it to inoculate 3 mL LB-CTK liquid media to grow over night, two times, back to back. This was to ensure the loss of the pBT-S2 plasmid, so that the pTRG-partner plasmid could be isolated. Once quantitated, I used the pTRG-partner plasmids to perform 32 different cotransformations (one for each putative positive) using the pTRG-partner plasmid, and pBT-S2. This cotransformation ensures that the putative positives are true positives. I put the plate in a 30°C incubator over night, and saw that every transformation produced colonies. This meant that each positive was confirmed to be an actual positive.

Now that they are positives, the goal is to eliminate a family of false positives. This family of false positives are false positives that arose from an unwanted interact between the DNABP and RNAP. To test for this interaction, I began to perform the 32 cotransformations, but only transforming with pBT (no S2) and the pTRG-partner plasmids. I have currently performed 8 of the 34 cotransformations (the

cotransformations were performed exactly the same way as before, except that this time I only transformed with pBT instead of pBT-S2), and each cotransformation resulted in the production of colonies. The production of colonies while testing the interaction of pTRG-partner and pBT means that the DNABP produced by the pBT plasmid interacted with the RNAP produced by the pTRG-partner plasmid. Basically, the 8 putative positives are branded as false positives.

Conclusion

The components needed for the two-hybrid experiment have been constructed, and the preliminary screening has begun. To date, there are approximately 100 possible positives that came from the pBT-S2 + pTRG-library experiment, where 34 of these positives have been screened again testing for an interaction between the isolated pTRG and pBT-S2. All 34 positive colonies gave positive results for an interaction. Which confirms that the pTRG-plasmids isolated from those 34 clones are positive results. Then, 8 of the 34 putative positives were tested to see if they were apart of a family of false positives, by testing an interaction between pBT and the pTRG-partner plasmid isolated from the putative positives. All 8 of the tested putative positives were false positives, but 24 clones remain to be screened. What is left is to test the remaining partner clones to see if they too belong to the family of false positives. If the additional screening results in the absence of colonies once a cotransformation is performed with pBT and the isolated pTRG-partner plasmid, then the pTRG-partner plasmid needs to be sequenced. With the assistance of computer-aided searches, the sequences will result in the identification of partners that interact with S2 in *E. coli*.

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Texas A&M University (96 credit hours), genetics and biochemistry majors, math and economics minors, 3.781 cumulative GPA, College Station TX
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Professional Experience:

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Research included using the bacterial two-hybrid to characterize the S2 protein of equine infectious anemia virus at the molecular level. The project required knowledge and use of SDS-PAGE, western blot analysis, agarose gel electrophoresis, cloning, sequencing, PCR, buffer production, plasmid isolation and manipulation.

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Undergraduate researcher, Advisor **Dr. Ellen Collisson**

Research included using cloning techniques to add ubiquitin and growth hormone tags to plasmids for DNA vaccine production. The research helped prepare the plasmids used in a DNA vaccine experiment producing DNA vaccines against feline immunodeficiency virus (FIV), infectious bronchitis virus (IBV), and reticuloendothelial virus (REV). The project required knowledge and use of ELISA, particle capture ELISA, SDS-PAGE, southern blot analysis, cloning, sequencing, PCR, buffer production, and restriction digest experiments.

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The project was to use restriction digests on genomic DNA of several different species of fusarium, an aflatoxin producing fungus, along with southern blot analysis to see if the genes involved in aflatoxin production were clustered together within the genome. The project required skills in restriction enzyme digests, southern blot analysis, radio-labeling methods, agarose gel electrophoresis, buffer production, and sequencing using BIG-DYE.

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